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Short communication

Identification of the Bcl-2 family protein gene BOK from orange-spotted grouper (*Epinephelus coioides*) involved in SGIV infection

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ABSTRACT

Apoptosis plays vital roles in many physiological process and immune response. BOK is one of the central regulators in apoptosis. In this study, a new BOK homolog (Ec-BOK) was cloned and characterized from Orange-spotted grouper, *Epinephelus coioides*. Ec-BOK encoded a 210 amino acid peptides which shared 97% identity to *Stegastes partitus* BOK protein, contained four BH domains and one transmembrane region. Ec-BOK widely expressed in all analyzed tissues with the higher expressions in kidney and spleen. Its expression level was up-regulated after SGIV infection *in vitro*. Further analysis revealed that overexpression of Ec-BOK inhibited viral genes transcriptions and virus replication in fish cell. Our findings suggested that Ec-BOK might play a role in the immune response against virus.

1. Introduction

Apoptosis or programmed cell death (PCD) is a high conserved cellular process that plays a crucial role in multiple biological processes including development, homeostasis and immunity [1,2]. Apoptosis can be mainly modulated by two apoptotic pathways: intrinsic pathway or extrinsic pathway [2]. The members of B-cell lymphoma 2 (Bcl-2) protein family act as key regulators of intrinsic pathway through modulating permeabilization of the outer mitochondrial membrane, which leading to apoptogenic factor released or activated [3–5].

Bcl-2 family homologies possess characteristics protein sequence called Bcl-2 homology (BH1, BH2, BH3, and BH4) domains, can be divided into two groups functionally: pro-apoptotic proteins (such as Bax, Bak, Bid and Bim), or anti-apoptotic proteins, exemplified by Bcl-2, Bcl-xL and Bcl-w [2,4]. To date, Bcl-2 family proteins have been well described from vertebrate to invertebrate [6,7]. Bcl-2 related ovarian killer (BOK) is an important pro-apoptotic protein of Bcl-2 family that was initially identified from rat ovarian and involved in erasing cancer cells by activating apoptosis [8].

Orange-spotted grouper, *Epinephelus coioides*, a marine commercial fish widely farmed in China and Southeast Asian counties. Recent years, the outbreak of viral infectious diseases caused by Singapore grouper iridovirus (SGIV) and nervous necrosis virus (VNN) have caused huge economic losses in grouper aquaculture [9–11]. Induction of programmed cell death in different fish cells after SGIV infection have also been well-documented [12,13]. While the roles of Bcl-2 family proteins during viral invasion remained largely unknown. In this study, a BOK gene was isolated and characterized from grouper, *Epinephelus coioides* (Ec-BOK). These works

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Table 1
Sequences of primers used in this study.

Primers	Sequences (5′-3′)
EcBOK3′GSP1	CTGGGTGGATGTAACAAAGTGCGTGGT
EcBOK3′GSP2	TGGCTGGTGTGTGCCGTCTGTGCCT
EcBOK 5′NGSP1	CACTTTGTTACATCCACCCAGCCACCT
EcBOK 5′NGSP2	GGAGGTCAGGCTTTTACGGACAAACT
pCDNA-EcBOK-F	CCGCTCGAG ATGGAGATGTTGCGCCGCTCTTCT
pCDNA-EcBOK-R	GGAATTC CTTCTCCCTGAAGAGGTATAACACAAT
RT-EcBOK-F	GGGGGAAGGTGGTGTCTTTGTA
RT-EcBOK-R	CAGTGAGAGCGGAAGCTGGGAT
RT-18S-F	ATTGACGGAAGGGCACCACCAG
RT-18S-R	TCGCTCCACCAACTAAGAACGG
RT-Actin-F	TACGAGCTGCCTGACGGACA
RT-Actin-R	GGCTGTGATCTCCTTCTGCA
RT-ORF115-F	CGGAAAGAACACAGATAACGG
RT-ORF115-R	AAAAAACACATGGCTTGCAAA
RT-ORF072-F	GCACGCTTCTCTCACCTTCA
RT-ORF072-R	AACGGCAACGGGAGCACTA
RT-ORF049-F	ATGTACGTATACCCCGCAAT
RT-ORF049-R	TCATTTTTTTTGCCTAA
RT-ORF086-F	ATCGGATCTACGTGGTTGG
RT-ORF086-R	CCGTCGTCGGTGTCTATTC

would contribute to better understand the role of fish BOK in response to virus infection.

2. Materials and methods

2.1. Cell lines and virus

Grouper spleen cells (GS) and fathead minnow (FHM) epithelial cells were cultured in Leibovitz's L15 medium and M199 medium containing 10% fetal bovine serum (FBS, Invitrogen, USA) at 25 °C, respectively [12]. Propagation of SGIV was performed as described previously [14].

2.2. RNA extraction and cDNA synthesis

Total RNA from orange-spotted grouper tissues was isolated using TRIzol reagent (Invitrogen) according to manufacturer's protocol, and the total RNA of cell samples was extracted by SV Total RNA isolation System (Promega). The total RNA was detected by electrophoresis on 1% agarose gel. The RNA from sampled tissues was used for cDNA synthesis through M-MLV Reverse Transcriptase (Promega, USA). The spleenic RNA was used for templates synthesis of rapid amplification of cDNA ends (RACE) through cDNA Amplification Kit (Clontech, USA).

2.3. Rapid amplification of cDNA ends (RACE) of Ec-BOK cDNA

The primers used for RACE PCR were designed according to the EST sequence of the transcriptome library (Accession No. SRA040065.1) established in our laboratory, the 5′and 3′ ends of the Ec-BOK cDNAs were amplified with a SMART RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol. The sequences of the primers used were listed in Table 1. The RACE PCR condition and assembly of Ec-BOK cDNA were performed as described previously [15].

2.4. Bioinformatics analysis of Ec-BOK

The nucleotide and predicted amino acid sequences of Ec-BOK (Accession No. KU569163) were analyzed using Genetyx7.0 software. The similarities of Ec-BOK with other BOK proteins were analyzed using the BLASTP search program at the NCBI www.ncbi.nlm.nih.gov/blast. Multiple-sequence alignment of the reported BOK amino acid sequences was performed using ClustalX2.0 and a phylogenetic tree was constructed using the MEGA 5.0 software.

Table 2
GenBank accession numbers of BOKs used in this study.

Protein	Accession no.
Epinephelus coioides	KU569163
Stegastes partitus	XP_008303220
Larimichthys crocea	XP_010738088
Maylandia zebra	XP_004552920
Oreochromis niloticus	XP_003444315
Takifugu rubripes	XP_003973834
Esox lucius	XP_010894739
Danio rerio	NP_001003612
Oryzias latipes	XP_011472219
Gallus gallus	AAG01182
Xenopus laevis	NP_001139563
Mus musculus	NP_058058
Homo sapiens	NP_115904

2.5. Tissue distribution of Ec-BOK

Total RNA was extracted from liver, spleen, head kidney, brain, intestine, heart, skin and muscle from healthy fish using TRIzol Reagent (Invitrogen, USA) according to manufacturer's instructions. Expression of Ec-BOK in various tissues was determined by qRT-PCR using RT primers shown in Table 1. The PCR conditions were applied as follows: 94 °C for 5 min, followed by 40 cycles of 5s at 94 °C, 10 s at 55 °C and 15 s at 72 °C. 18S was used as control with primers RT-18S-F and RT-18S -R (Table 1). The specificity of the PCR amplification for primers was verified from the dissociation curves. PCR amplification efficiency was identified according to the methods described in Ref. [16]. Relative gene expression was analyzed using $2^{-\triangle\triangle Ct}$ method [17].

2.6. Expression profile of Ec-BOK after SGIV infection in vitro

GS cells were grown in L15 medium containing 10% FBS, at 25 °C. For virus challenge *in vitro*, GS cells were infected with SGIV. The cells were harvested at 0, 6, 12, 24 and 48 h post infection for further analysis. The qRT-PCR analysis was performed as Section 2.5.

2.7. Effect of Ec-BOK overexpression on viral gene transcription

To detect the role of Ec-BOK on SGIV infection, we cloned Ec-BOK ORF and inserted into pcDNA3.1(+) vector using corresponding primers (Table 1). The constructed recombinant plasmid (Designated as pcDNA-Ec-BOK) was confirmed by sequencing for further studies. The plasmids pcDNA-EcBOK and pcDNA3.1(vector control) were transfected into FHM cells, respectively. After transfection for 24 h, cells were infected with SGIV. Subsequently the uninfected and SGIV-infected cells were collected at 36 h post infection (p.i.) for further analysis. To examine the level of SGIV replication, the transcriptions of viral genes ORF 072, ORF 049, ORF 086 and ORF 115 were detected